AD_______________

Award Number: W81XWH-06-1-0186

TITLE: Copine-I: Modulator of NF-kappa B Transcription and Prostate Cancer Survival

PRINCIPAL INVESTIGATOR: Marty W. Mayo, Ph.D.
Carl Creutz, Ph.D.

CONTRACTING ORGANIZATION: University of Virginia
Charlottesville, VA 22904

REPORT DATE: January 2007

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE
01-01-2007

2. REPORT TYPE
Annual

3. DATES COVERED
15 Dec 2005 – 14 Dec 2006

4. TITLE AND SUBTITLE

Copine-I: Modulator of NF-kappa B Transcription and Prostate Cancer Survival

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0186

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Marty W. Mayo, Ph.D.
Carl Creutz, Ph.D.

Email: mwm3y@virginia.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Virginia
Charlottesville, VA 22904

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
NF-κB is a dynamic transcription factor that regulates important biological processes involved in cancer initiation and progression. This is especially true in prostate cancer (PCa) where dysregulated NF-κB activity is directly associated with PCa relapse. Identifying regulators that control the half-life of NF-κB is important to understanding molecular processes that control the duration of transcriptional responses. In this the initial funding period, we identify Copine-I, a calcium phospholipid binding protein, as a novel repressor that physically interacts with p65 to inhibit NF-κB transcription. siRNA knockdown of Copine-I increases tumor necrosis factor (TNF) α-stimulated NF-κB transcription, while Copine-I expression blocks endogenous transcription. Our work provides evidence that Copine-I regulates the half-life of NF-κB transcriptional responses through a novel mechanism that antagonizes typical polyubiquitination of p65.

15. SUBJECT TERMS
Prostate cancer, NF-kappa B, transcription, cell survival, Copine-I, ubiquitin-dependent degradation, apoptosis, chromatin, acetylation, phosphorylation, proto-oncoprotein, tumor suppressor protein, nuclear export, nuclear import, extracellular matrix, tumor

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRMC
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	7	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

DOD Idea Development Award PC050549
(PI: Marty W. Mayo, Co-PI: Carl E. Creutz)

Introduction:
Androgen-ablation is initially an effective form of therapy for prostate cancer (PCa). However, some patients suffering from late-stage PCa often display a recurrence of the disease. One of the intrinsic properties of the recurrent androgen-independent PCa is an exquisite resistance to apoptosis. The androgen-independent PCa cells exhibit resistance to apoptotic cues mediated by death receptor ligands, loss of cellular attachment, as well as chemotherapy-induced cell death (1). In addition to losing functional androgen-receptor signaling pathways, many androgen-independent PCa cells display elevated NF-κB transcriptional activity. In most forms of cancer there are multiple avenues by which carcinomas arise. However, several laboratories have shown that NF-κB activity is dysregulated not only in PCa cells, but that the activation status of this transcription factor may predict the overall responsiveness of the disease to conventional anti-neoplastic therapies (2-5). To understand better how PCa cells become resistant to apoptotic stimuli, it is important to characterize the molecular pathways that control NF-κB-transcription and cell survival. Having previously established that acetylation of p65 at K310 is a prerequisite for full NF-κB transcription (6-8), our laboratory has developed an α-p65(AcK310) antibody and preliminary evidence that this "activation mark" is dysregulated in late-stage human PCa tumors. In order to understand mechanistically why PCa cells display dysregulated acetylated p65, we have identified a novel, naturally occurring, regulator of NF-κB transcription called Copine-I. Copine-I is a calcium-binding protein of previously unknown function. Preliminary data presented in this proposal indicate that Copine-I is an NF-κB regulated gene product that normally functions to control the half-life of the p65 subunit of NF-κB. The overall goal of this proposal is to understand the mechanisms by which Copine-I regulates p65 transcriptional activity and to determine whether Copine-I expression provides a pro-survival signal to androgen-independent PCa cells. To address the hypothesis described above, two aims will be explored. Aim 1 will elucidate the mechanisms by which Copine-I controls p65 turnover. Aim 2 will determine the role of Copine-I as an anti-apoptotic protein. Knowledge obtained from these studies will aid not only in our understanding of how NF-κB becomes dysregulated in PCa cells, but will potentially identify Copine-I as an important regulator of cell survival decisions. This work will potentially impact the way PCa is diagnosed and treated.

Body:
In this the first year of the award, we have made significant progress in all of the original aims of this proposal.

Progress on Aim 1: Experiments described in Aim 1 were to elucidate the mechanisms by which Copine-I controls p65 turnover. We found that Copine-I is able to inhibit NF-κB transcription mediated by all of the transcriptionally active components of NF-κB, including RelA/p65, RelB and cRel (Fig 1).

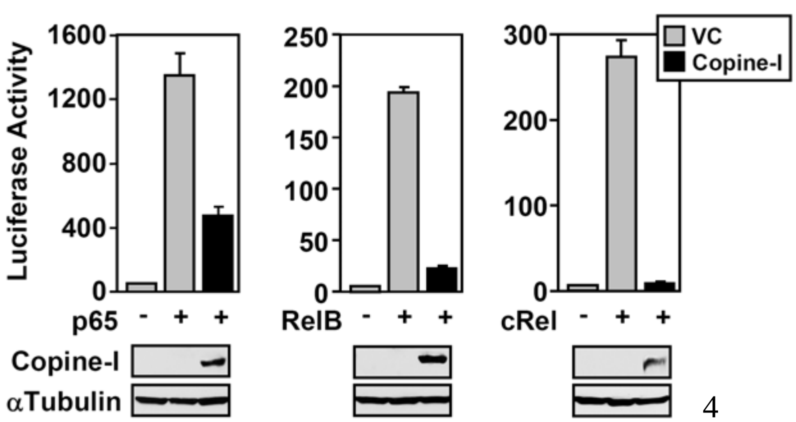

Figure 1: HEK 293T cells were co-transfected with the 3xκB-luciferase reporter and plasmids encoding empty vector (-), p65, RelB, or cRel with and without co-transfection of copine-I expression vector. Cells were harvested after 24 hours. Western blot analysis confirms expression of copine-I. Tubulin expression serves as a protein loading control. All luciferase assays were performed in triplicate and were repeated in three independent experiments. The mean ± standard deviations (SD) are indicated.

This is biologically relevant in PCa cells since a knockdown of Copine-I expression results in an increase in NF-κB gene expression following TNFα addition (Fig 2A). Copine-I interacts with RelA/p65 at an endogenous level (Fig 2B). Copine-I binds to the N-terminus of RelA/p65 (Fig 2C).

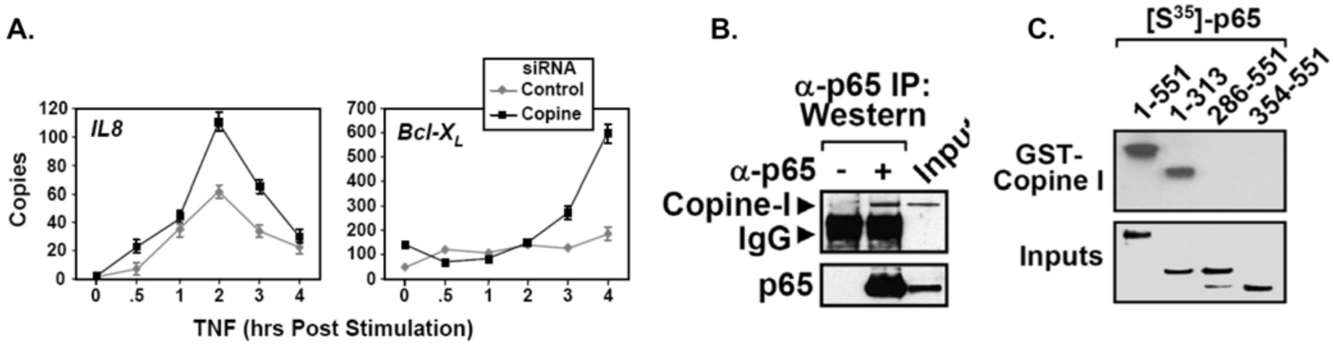

Figure 2: A) Message levels of NF-κB-regulated genes increase in the absence of copine-I. DU145 cells were transfected with copine-I siRNA or control siRNA. QRT-PCR was performed in triplicate with primers specific to *IL-8* or *Bcl-X$_L$* genes. The relative levels of mRNA expression were normalized to the housekeeping gene *HPRT*. **B)** Endogenous Copine-I interacts with p65. Total input (10%) protein is shown. **C)** *In vitro* translated [^{35}S]-p65 labeled proteins were pulled down with either GST or GST-copine-I. [^{35}S]-labeled p65 proteins were detected by autoradiography. Coomassie stained gels confirm the presence of GST proteins.

To better understand how Copine-I regulates NF-κB transcription, we examined which domains within Copine-I are required to repress RelA/p65 transactivation potential. We found that Copine-I dimerizes through its C2 domain and that the A domain of Copine-I was responsible for inhibiting NF-κB transcriptional activity (Fig 3A). Moreover, we found that membrane localization of Copine-I (Myr-copine) disrupts the ability of Copine-I to repress NF-κB transcription, as measured by quantitative real-time PCR (Fig 3B). Copine-I acts directly on the p65 component of NF-κB, rather than disrupting IKK-mediated signaling pathways. Future directions outlined in Aim 1 will elucidate mechanistically how Copine-I inhibits p65 transactivation potential. Although Copine-I was originally proposed to potentiate p65 turnover through ubiquitin-dependent mechanisms, further experimentation clearly indicates that Copine-I functions in a unique manner to block NF-κB transcription. This is evident by the fact that Copine-I expression stabilizes a truncated form of p65, suggesting that rather than potentiating p65 turnover Copine-I promotes stabilization.

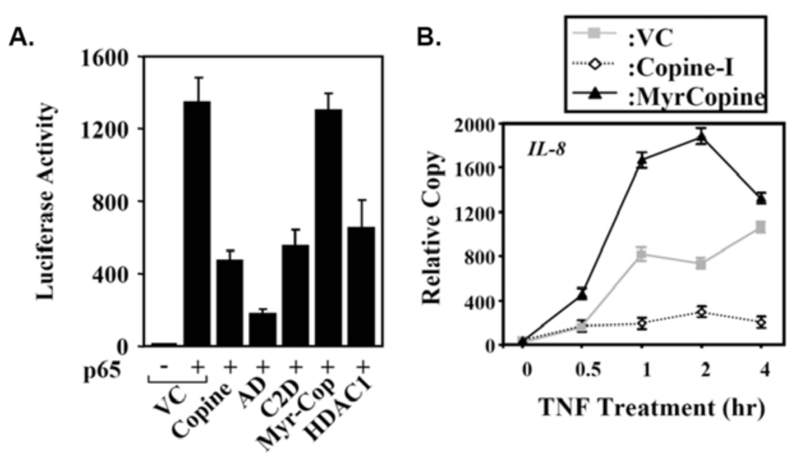

Figure 3: A) Loss of NF-κB transcriptional activity in HEK 293T cells expressing various copine-I expression plasmids. **B)** Localization of copine-I to the membrane increases *IL-8* mRNA expression. HEK 293:VC, HEK 293:copine-I, and HEK 293:myr-copine-I stable cell lines were treated with TNFα for the indicated times. Total RNAs were harvested and QRT-PCR analysis was performed using the *IL-8* primer set.

Progress on Aim 2: Experiments described in Aim 2 will determine the role of Copine-I as an antiapoptotic protein. Evidence provided thus far indicates that Copine-I negatively regulates NF-κB transcription. NF-κB has been shown to block many different forms of apoptotic stimuli, including those mediated by loss of attachment to the extracellular matrix, a form of apoptosis referred to as anoikis (9). To determine whether Copine-I could sensitize cells to anoikis, we created cell lines stably expressing either Copine-I or Myr-copine. Although control cells displayed increased apoptosis following detachment, cells stably expressing Copine-I were more resistant to anoikis, displaying reduced levels of nucleosome and caspase-3 activity (Fig 4). In contrast, cells stably expressing mislocalized Copine-I (Myr-copine) displayed increased induction of apoptosis following loss of cell attachment. Together these experiments indicate that cells expressing Copine-I cells are more resistant to apoptotic cues despite the fact that these cells displayed reduced NF-κB transcription.

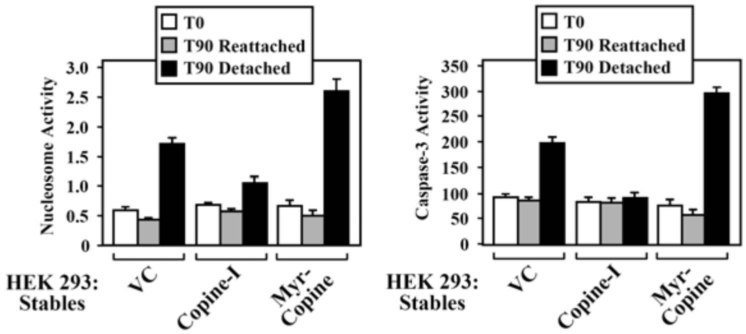

Figure 4: HEK 293:VC, HEK 293:copine-I, and HEK 293:myr-copine-I stable cells lines were either left untreated (T0), or trypsinized and re-plated in serum free media on tissue culture treated dishes (T90 Reattached) or on polyHema coated dishes (T90 Detached). After 90 minutes cells were harvested and apoptosis was measured by nucleosome and Caspase-3 activity assays.

Key Research Accomplishments:

- Human PCa cells display elevated Copine-I protein expression.
- Copine-I is a natural inhibitor of NF-κB transcription.
- Copine-I functions by directly interacting with the p65 subunit of NF-κB.
- Mislocalization of Copine-I to the cell membrane disrupts endogenous Copine activity, which results in increased NF-κB transcriptional activity.
- Although Copine-I blocks NF-κB transcriptional activity, Copine-I expression prevents apoptosis induced in response to cell detachment and TNFα.

Reportable Outcomes:

Based on the preliminary data generated thus far, we are currently preparing our first manuscript describing the role of Copine-I as a novel repressor of NF-κB transcription.

Conclusion:

Work from our laboratory has also recently shown that the p65 component allows PCa cells to remain resistant to apoptosis when grown in suspension. Such model systems have been important in demonstrating that resistance to apoptosis following loss of attachment is critical for the development of metastasis of PCa cell to bone (10,11). This work may potentially lead to the development of unique cancer therapies that target NF-κB transcription and components of these signaling pathways. Although we have found that Copine-I is a repressor of NF-κB, the exact mechanism by which Copine-I functions needs further examination. Experimental evidence suggests that Copine-I can modify the p65 component of NF-κB in one of two ways. Chromatin-bound p65 is cleaved into a 55 KD sized protein which no longer contains the N-terminal DNA binding domain. In this way the 55 KD protein acts as a dominant negative protein to unmodified full length p65 protein. However, Copine-I also seems to have another function. Ectopic expression of Copine-I results in posttranslational modification of p65. To our surprise, Copine-I modification was not through ubiquitin or ubiquitin-like modifications. Interestingly, Copine-I also rescues or blocks ubiquitin-dependent degradation of p65, suggesting that Copine-I may antagonize E3-mediated ubiquitin process. In support of this we have found that Copine-I interacts with JM1, an adaptor molecule which is an integral member of the Cullin/SOC3/COMD1 complex. This complex has recently been shown to be responsible for degradation of p65 (12). Thus, future experiments will elucidate whether Copine-I antagonizes this ubiquitin complex by the addition of additional posttranslational modifications. In collaboration with Ezra Burstein's laboratory (University of Michigan), we will determine if the 55KD fragment of p65 is resistant to polyubiquitination mediated by the Cullin/SOC3/COMD1 complex using in vitro and in vivo ubiquitin assays. Since COMD1 interacts with p65 in the first 180 amino acids, we predict that Copine-I dependent processing of p65 will now be resistant to polyubiquitination. In summary, these experiments will determine the molecular mechanism by which Copine-I functions as a novel regulator of NF-κB transcription and cell survival in PCa cells.

References:

1. Denmeade, SR, Lin, XS, and Isaacs, JT. 1996. Role of programmed (apoptotic) cell death during the progression and therapy for prostate cancer. Prostate 28:251-265.
2. Palayoor,S.T., M.Y. Youmell, S.K. Calderwood, C.N. Coleman,, and B.D. Price. .1999. Constitutive activation of IKKα and NF-κB in prostate cancer cells is inhibited by ibuprofen. Oncogene 18,7389-7394.
3. Gasparian,A.V., Yao,Y.J., Kowalczyk,D., Lyakh,L.A., Karseladze,A., Slaga,T.J., and Budunova,I.V. 2002. The role of IKK in constitutive activation of NF-κB transcription factor in prostate carcinoma cells. J Cell Sci 115, 141-151.
4. Chen, C.D. and C.L. Sawyers. 2002. NF-κB activates prostate-specific antigen expression and is upregulated in androgen-independent prostate cancer. Mol. Cell. Biol. 22, 2862-2870.
5. Shukla, S, MacLennan, GT, Fu, P, Patel, J, Marengo, SR, Resnick, MI, and Gupta, S. 2004. Nuclear factor- κB/p65 (Rel A) is constitutively activated in human prostate adenocarcinoma and correlates with disease progression. Neoplasia 6:390-400.
6. Hoberg,J.E., Yeung,F., and Mayo, M.W. 2004. SMRT derepression by the IκB kinase α: A prerequisite to NF-κB transcription and survival. Mol Cell 16:245-255.
7. Yeung,F., Hoberg,J.E., Ramsey,C.S., Keller,M.D., Jones D.R., Frye,R., and Mayo,M.W. 2004. Modulation of NF-κB-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J. 23:2369-2380.
8. Hoberg,J.E., Popko, A.E., Ramsey, C.S. and Mayo, M.W. 2006. IκB kinase α-mediated derepression of SMRT potentiates acetylation of RelA/p65 by p300. Mol. Cell. Biol. 26:457-471.
9. Frisch SM, and Francis H. 1994. Disruption of epithelial cell-matrix interactions induces apoptosis. J. Cell Biol. 124:619-626.
10. Rennebeck, G., M. Martelli, and N. Kyprianou. 2005. Anoikis and survival connections in the tumor microenviroment: Is there a role in prostate cancer metastasis? Cancer Res. 65:11230-11235.
11. Chung L.W., A. Baseman, V. Assikis, HE. Zhan. 2005. Molecular insights into prostate cancer progression: the missing link of tumor microenviroment. J. Urol.173:10-20.
12. Maine GN, Mao X, Komarck CM, Burstein E. 2007. COMMD1 promotes the ubiquitination of NF-κB subunits through a Cullin containing ubiquitin ligase. EMBO J. 26:459–467.

Appendices: none included